

Developmental expression of G proteins that differentially modulate adenylyl cyclase activity in mouse brain

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Changes in the relative abundance of the G protein α subunits were observed during early mouse development. $G_{s\alpha}$ was almost exclusively present as a large form ($G_{s\alpha 1}$) in prenatal brain. Postnatally with a substantial increase in Gpp[NH]p stimulated adenylyl cyclase activity, the small form ($G_{s\alpha 2}$) increased in amount while $G_{s\alpha 1}$ decreased. These results suggest that the $G_{s\alpha 2}$ may be the more effective cyclase activator and that changes in alternative splicing are developmentally regulated. G_{i1} and G_o appeared before birth whereas G_{i2} developed postnatally. Opiate stimulation of GTPase and inhibition of adenylyl cyclase were fully expressed prenatally.

Development (Mouse brain); G protein; Alternative splicing; Adenylyl cyclase; Opiate receptor

1. INTRODUCTION

The heterotrimeric G proteins mediate signal transduction across cell membranes; they are activated by extracellular receptors and subsequently modulate the activities of intracellular proteins responsible for second messenger production or ion channel control [1–3]. Adenylyl cyclase is subject to stimulatory control by receptors acting through G_s and inhibitory modulation by those working through a G_i . Four different G_s α -subunits of two major size classes are known to be generated by alternative splicing of a single precursor gene [4–6]. There are also a number of G_i species, many of which are products of different genes [7–9]. The major G_i species found in brain are G_{i1} and G_{i2} [10–12]. One or more of these proteins is responsible for coupling inhibitory receptors to adenylyl cyclase [1–3]. Here we report studies in which we measured the amounts of the G protein α -subunits present during pre- and early postnatal development of the mouse brain together with Gpp[NH]p stimulation of adenylyl cyclase as a measure of G_s activity, and opiate inhibition of adenylyl cyclase and stimulation of low K_m GTPase to address G_i functionality.

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Abbreviations: G proteins (G_s , G_{i1} , G_{i2} and G_o), guanine nucleotide regulatory proteins; E, embryonic day; EGTA, [ethylenedis-(oxymethylene)trilo]tetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Gpp[NH]p, guanylyl-5'-yl-imidodiphosphate; TBST, 0.05% Tween in Tris-buffered saline

2. MATERIALS AND METHODS

2.1. Animals and tissue preparation

Adult male and female NIH white mice (NC1, Frederick) were mated by placing one male with four females for 12–14 hours; males were then removed and this moment was considered as embryonic day 0.5. Pregnant females were euthanized by cervical dislocation followed by decapitation at different stages of the embryonic development; similar procedures were used in postnatal days. Brains were homogenized in 20 vols of 0.32 M sucrose. Samples were centrifuged at $1000 \times g$ for 10 min; the supernatant suspensions were centrifuged at $48000 \times g$ for 15 min and the pellets were resuspended and washed twice in 50 vols of 2 mM Tris (pH 7.4), 2 mM EGTA.

2.2. Western blots

Membrane fractions prepared as above were treated as described by Sternweis and Robishaw [10]; samples were separated by SDS-PAGE [13] and transferred to nitrocellulose membranes using a Sartorius semi-dry electroblotter [14]. Blots were incubated at room temperature with 1% gelatin in TBST (20 mM Tris-HCl (pH 7.5), 500 mM NaCl and 0.05% Tween 20) for 1 h, washed 3 times for 10 min with TBST, and incubated with the appropriate antibody for 2 h. G_s was reacted with antibody RM (1:750) [15] or in other experiments by anti- G_s antibody 584 (1:1000) [2] was used with similar results. This antiserum recognized proteins with apparent molecular weights of 46 000 for $G_{s\alpha 1}$ and 42 300 for $G_{s\alpha 2}$, in a 10–12.5% polyacrylamide gel using high molecular weight rainbow markers (Amersham). G_{i1} was quantitated with antibodies AS/7 (1:100) [16] and LD (2 μ g/ml) [17]; G_{i2} by antibodies AS/7 and LE (5 μ g/ml) [16]; G_o by antibody $G_o/1$ [12]. Blots were washed 3 times over 10 min with TBST and then incubated with [¹²⁵I]Protein A (100 000 cpm/ml) for 30 min followed by 3 washes of 10 min. Blots were then exposed to Kodak AR2 film for 4–16 h. The autoradiograms were analyzed by densitometry of images generated by a television camera interfaced with a Macintosh II computer using the program IMAGE developed by Wayne Rasband [18]. The linearity of the signal density and film saturation were monitored by using different amounts of membrane protein on the same gel.

2.3. Adenylyl cyclase and GTPase measurements

For Gpp[NH]p stimulated adenylyl cyclase, pellets were resuspend-

ed in 2 mM Tris (pH 7.4), 2 mM EGTA at approximately 5 mg prot./ml. The reactions contained 50 μ l of membranes in a final volume of 250 μ l in the presence of 25 mM Tris-HCl (pH 7.4), 1 mM isobutylmethylxanthine, 2 mM $MgCl_2$, 0.4 mM EGTA, 10 mM creatinephosphate, 25 U of creatine kinase and 0.5 mM ATP. After 10 min at 37°C, the reaction was stopped by the addition of 10 μ l of 0.5 M EDTA and boiling for 5 min. cAMP was measured by radioimmunoassay with a commercially available kit (NEN). *Opiate sensitive adenylyl cyclase* activity was measured by a modification [19] of the method of Salomon et al. [20]. For these analyses membranes were prepared from brain homogenates in 0.32 M sucrose at 1–2 mg prot./ml. The homogenate was centrifuged at 1000 $\times$ g for 10 min and the supernatant suspension was centrifuged for 20 min at 48000 $\times$ g. The pelleted membranes were suspended at 1–2 mg prot./ml in 0.32 M sucrose, 10 mM Tris-HCl (pH 8) and stored in aliquots at –70°C. 20 μ g protein were used in each assay. Etorphine effects were shown to be reversed by naloxone. Other opiates tested, including μ - and δ -selective opioid peptides, were less effective than etorphine with developing mouse brain membranes.

Low K_m GTPase activity was measured by a modification [21] of the method of Cassel and Selinger [22]. GTPase measurements were performed with 5 μ g protein per sample.

2.4. Protein assay

Protein content was determined by a dye binding assay [23] from Bio-Rad or by the method of Lowry et al. [24].

3. RESULTS AND DISCUSSION

The amounts of G protein α -subunits present in brain membranes were measured by quantitative immunoblotting. Antibodies specific for G_s recognized the large and small forms of the protein (Fig. 1A). Interestingly, the amount of G_{s-1} was more than twice that of adult at embryonic days 14.5–16.5, whereas G_{s-5} was less than 10% of adult prior to E18.5 (Fig. 1B). The amount of G_{s-1} decreased as G_{s-5} increased late in development. G_s levels were close to those of the adult at postnatal day 14, at which time the concentrations of the large and small forms in whole brain membranes were similar. The reciprocal relationship between the amounts of the two species found during development suggests that a change in the alternative splicing mechanism occurred late in development. A similar finding has recently been reported in brown adipose tissue, in which it was established that the ratio of the protein forms directly reflects that of the mRNA [25]. It will be of interest to see whether the alternatively spliced forms of G_o [26,27] develop in parallel to those of G_s .

We measured the extent of guanine nucleotide stimulation of adenylyl cyclase in brain membranes of various developmental stages. The GTP analogue Gpp[NH]p stimulated adenylyl cyclase approximately 3-fold between embryonic days 12.5 and 16.5 but at E18.5 the amount of stimulation started to rise towards the adult level of 5 to 6-fold (Fig. 2). Reconstitution experiments with individually expressed G_{s-1} and G_{s-5} forms have shown that the larger species were activated more rapidly, but that they also had a higher intrinsic GTPase activity [28,29]. Our results are consistent with several observations that most hormonal stimulation of adenylyl cyclase develops postnatally [30–32], and sug-

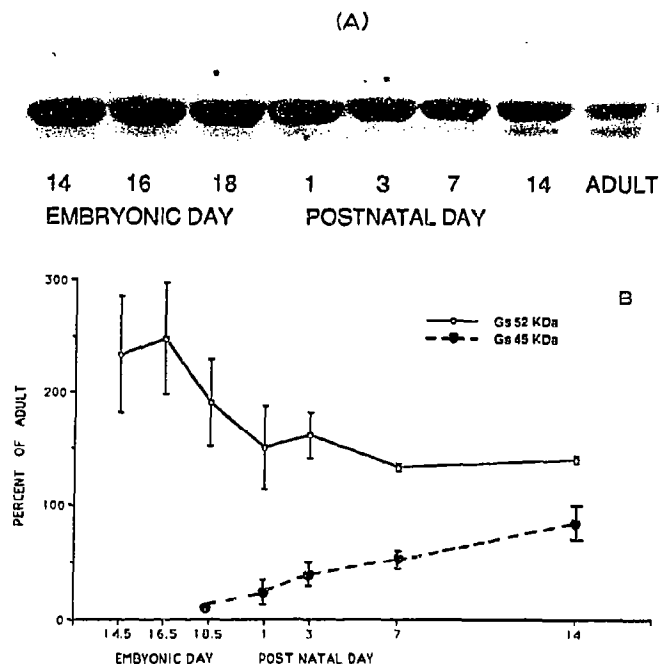


Fig. 1. A. Immunoblot analysis of G_{s-1} and G_{s-5} in mouse brain membranes (25 μ g protein) from pre- and postnatal development. Samples were subject to a 12.5% SDS-PAGE system and transferred to nitrocellulose membranes as described in section 2. For this blot the G_s RM antibody (1:750) was used. B. Densitometric analysis of autoradiograms showing the ontogeny of G_{s-1} and G_{s-5} subunits in mouse brain membranes. The data represent mean $\pm$ SD of at least 3 separate gels from no less than 3 different pools of animals at each age.

gest that G_{s-5} species may be primarily associated with hormonal activation of adenylyl cyclase, as shown for HIT cells [33]; whereas G_{s-1} species may serve additional roles as well [34].

The α -subunits of G_{i1} and G_o [35,36] showed an almost linear increase throughout the prenatal development of the brain (Fig. 3A and B) whereas G_{i2} was not

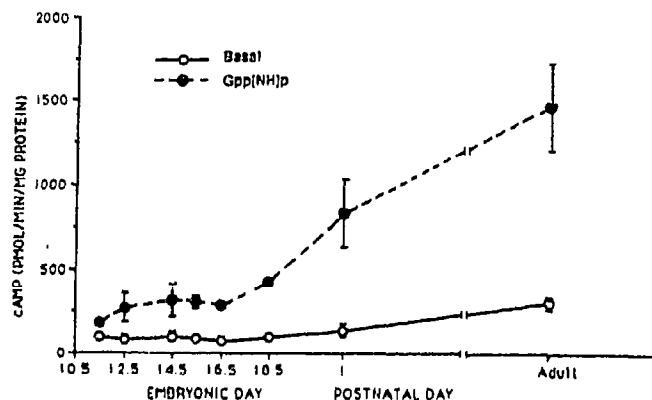


Fig. 2. Development of adenylyl cyclase activity in synaptosomal membrane preparation of mouse brain. cAMP formation was measured in the absence (○—○) or presence of 10 μ M Gpp[NH]p (●—●). Each point is the mean $\pm$ SD of 3 or 4 experiments performed in triplicate.

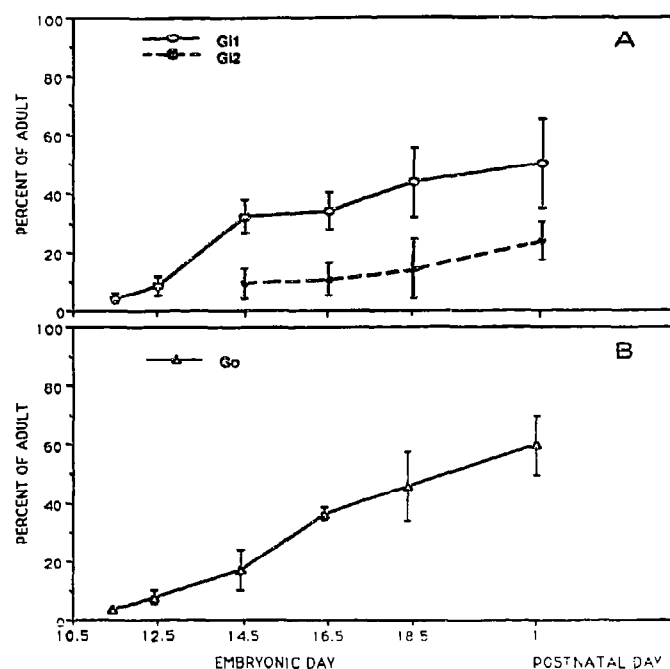


Fig. 3. Immunoblot analysis of the appearance of G₁₁ and G₁₂ (A) and G₀ (B) during early mouse brain development. Values are the mean $\pm$ SD of 2-6 determinations at each age.

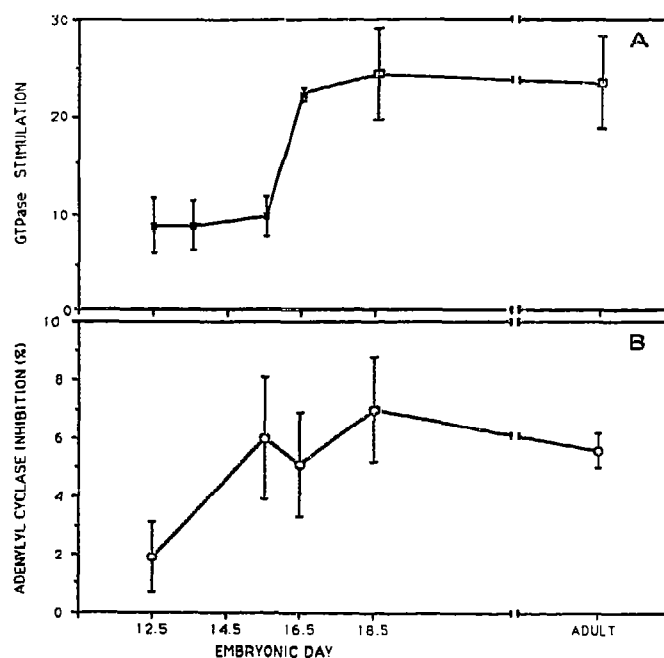


Fig. 4. Effects of 1 μ M etorphine upon low K_m GTPase (A) and adenylyl cyclase (B) activities of mouse brain membranes during development. Assays were performed in pentuplicate and the results are presented as mean $\pm$ SEM.

present in appreciable amounts prior to birth (Fig. 3A). To assess the functional significance of these developmental profiles, stimulation of GTPase activity by the potent, and receptor subtype unselective opiate etorphine was measured. Stimulation of GTPase was detectable at E12.5, and reached adult levels by E18.5 (Fig. 4A). This developmental profile did not correspond with that of any of the G proteins measured (Fig. 3) and suggests that it is the receptor which is limiting in early development. Several studies of opiate receptors in rodent brain [37-40] showed that μ -receptors are fully developed at E14.5 and that δ are found only after birth, but that κ -opiate receptors which are detectable by E14.5 reach adult levels by E16.5 [40]. Thus, there is a good correlation between the times of development of κ -receptors and of opiate stimulated GTPase activity. We do not know the nature of all of the opiate sensitive effector systems operating prenatally, but measurements of adenylyl cyclase inhibition of etorphine showed that inhibition was present as early as E12.5, and reached adult levels near E15.5 (Fig. 4B). It had been shown earlier that μ -opiate receptors account for only a small fraction of opiate inhibition of adenylyl cyclase in neonatal rat brain [40]. Thus, κ -opiate receptors, which are known to inhibit adenylyl cyclase in spinal cord [42], may mediate the bulk of opiate stimulation of GTPase and inhibition of adenylyl cyclase in developing brain. Interestingly, α_2 -adrenergic receptors which mediate inhibition of adenylyl cyclase [43], are also developed primarily during the prenatal period [44].

Adenylyl cyclase activity is controlled both by stimulatory and inhibitory hormone receptors. Our results suggest that the brain enzyme may be relatively refractory to stimulatory hormone action prior to birth because G_{s-s} has not yet developed, but that inhibitory hormone control of its activity is present. Thus, it may be important to keep cAMP levels low during this period of rapid cell division. Other activities of G_i-coupled receptors, such as the control of ion channels and phospholipid metabolism, may also play central roles in the early control of brain maturation.

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